Chapter 3
Applications of L-asparaginase
Chapter 3(A)

Deglycosylation of glycoprotein’s by L-asparaginase
5A.1. Introduction

Glycosylation is one of the most common post-translational modifications of proteins in eukaryotic cells. These glycoproteins are involved in a wide range of biological functions such as receptor binding, cell signalling, immune recognition, inflammation, and pathogenicity. Glycosylation is one of the major naturally occurring modifications of the covalent structure of proteins. Most secretory proteins become glycosylated as soon as the growing polypeptide chains enter the endoplasmic reticulum, before the final native-like folded state is attained. Mammalian glycoproteins contain three major types of oligosaccharides (glycans): N-linked, O-linked, and glycosylphosphatidylinositol (GPI) lipid anchors. N-Linked glycans are attached to the protein backbone via an amide bond to an asparagine residue in an Asn-Xaa-Ser/Thr motif, where X can be any amino acid, except proline. O-Linked glycans are linked via the hydroxyl group of serine or threonine. Variation in the degrees of saturation at available glycosylation sites results in heterogeneity in the mass and charge of glycoproteins. The biological functions of glycoproteins are well established but the role that carbohydrates play in these functions is, for the most part, unclear with contradictory reports frequently observed in the literature (Chu et al., 1978; Schulke and Schmid, 1988; Rudd et al., 1994; Kern et al., 1993; Bernard et al., 1983; Paul et al., 1986).

To study the structure and function of a glycoprotein, it is often desirable to remove all or just a selected class of glycans. This approach allows the assignment of specific biological functions to particular components of the glycoprotein. The removal of N-linked glycans from glycoproteins eliminates heterogeneity in matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS analysis. Also, removal of glycans may enhance or reduce the blood clearance rate and/or potency of a therapeutic glycoprotein. Although sites of potential N-glycosylation can be predicted from the consensus sequence Asn-Xaa-Ser/Thr, it cannot be assumed that a site will actually be glycosylated. Therefore the sites of glycan attachment must be identified and characterized by analytical procedures. Peptide-N-glycosidase F (PNGase F) is one of the most widely used enzymes for the deglycosylation of glycoproteins. The enzyme
releases asparagine-linked (N-linked) oligosaccharides from glycoproteins and glycopeptides. In the present study, OVA and IgY were chosen as a model glycoproteins for deglycosylation using L-asparaginase from *Cladosporium* sp. and a comparative study was performed using these two native proteins.

### 5A.2. Materials

#### 5A.2.1. Chemicals

Chicken ovalbumin (OVA) and Nonidet P-40 were obtained from Sigma (St Louis, MI). Immunoglobulin Y (IgY) was isolated from white leghorn chicken (layers) egg yolk. All other chemicals were of the highest analytical grade and purchased from standard chemical companies.

#### 5A.2.2. Reagents

##### 5A.2.2.1. Buffers

- **Sodium acetate buffer (pH: 4.0–6.0)**
  
  Sodium acetate buffer was prepared as given under section 4A.2.2.1.

- **Sodium Phosphate buffer (pH: 6.0–8.0)**
  
  Sodium Phosphate buffer was prepared as given under section 4A.2.2.2.

- **Carbonate buffer (pH: 8.0-10.5)**
  
  Carbonate buffer was prepared as given under section 4A.2.2.3.

##### 5A.2.2.2. Denaturation buffer

- **Sodium acetate buffer (pH 4.5)**

  **Components**
  
<table>
<thead>
<tr>
<th>Grams/litre (0.1M)</th>
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<tbody>
<tr>
<td>a). Sodium acetate</td>
</tr>
<tr>
<td>b). Acetic acid</td>
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</table>

  Solution a and b were mixed together to obtain required pH 4.5 and the volume was made upto 1 L with dist.H₂O.

- **Zinc Chloride (0.1 mM)**

<table>
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<th>Grams/litre</th>
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<tr>
<td>ZnCl₂</td>
</tr>
<tr>
<td>dist.H₂O</td>
</tr>
</tbody>
</table>
- β-mercaptoethanol 0.1% (v/v)
  β-mercaptoethanol : 0.1 g
  dist.H2O : 100 ml

5A.3. Methods

5A.3.1. Denaturing reaction conditions
Denaturation reaction was carried out according to Tarentino & Plummer, (1994) as follows:
1. 100 µg of glycoprotein was dissolved 18 µl H2O.
2. 2 µl of 10X Glycoprotein denaturing buffer (5A.2.2.2) was added to make a 20 µl total reaction volume.
3. Glycoprotein was denatured by heating reaction at 100°C for 10 minutes.
4. Denatured glycoprotein was chilled on ice and centrifuged for 10 seconds.
5. To the denatured glycoprotein reaction 5 µl reaction Buffer (15 mM sodium phosphate pH 6.3), 5 µl 10% Nonidet P-40 and 15 µl H2O were added.
6. 5 µl Deglycosylation enzyme L-asparaginase from Cladosporium sp. was added and mixed gently.
7. Reaction mixture was incubated at 37°C for 4 hours.
8. Release of oligosaccharides was monitored by observing altered protein band migration by SDS gel electrophoresis.

5A.3.2. Non-denaturing Reaction Conditions
Non-denaturing reaction was carried out according to Tarentino & Plummer, (1994) as below:
1. 100 µg of glycoprotein was dissolved in 40 µl H2O.
2. To the native glycoprotein 5 µl reaction buffer (15 mM sodium phosphate pH 6.3) was added.
3. 5 µl Deglycosylation enzyme L-asparaginase from Cladosporium sp. was added and mixed gently.
4. Reaction mixture was incubated at 37°C for 4 hrs.
5A.3.3. Influence of temperature on deglycosylation

Deglycosylation of ovalbumin and IgY using *Cladosporium* sp. L-asparaginase was carried at different temperatures 16, 20, 30, 45 and 50°C. Other reaction protocols are as given under 5A.3.1.

5A.3.4. Influence of pH on Deglycosylation

Deglycosylation of ovalbumin and IgY using *Cladosporium* sp. L-asparaginase was carried at different pH values from 5.0 through 10.0 using different buffers. Sodium acetate buffer was used for pH values from 4.0 to 6.0 (4A.2.2.1); phosphate buffer was used for pH values between 6.0 to 8.0 (4A.2.2.2) and Carbonate buffer was used for pH values from 8.0 to 10.0 (4A.2.2.3). Other reaction protocols are as 5A.3.1.

5A.4. Analytical

5A.4.1. Electrophoresis

The extent of carbohydrate removal was tested by Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate according to Laemmli, (1970) with a 5 % acrylamide concentration for the stacking gel and a 10 % acrylamide concentration for the separating gel. Glycoprotein sample was diluted in loading buffer (4A.2.2.7.6) to obtain a concentration of 1 mg protein/ml and heated for 5 min in a boiling water bath. 40 μl aliquots were loaded in duplicate to the wells. Run time was between 3 and 4 h at 80 V under constant current conditions. At the end of the migration, the gel was stained with Coomassie brilliant blue (4A.2.2.7.7.1), destained at room temperature using destaining solution (4A.2.2.7.8).

5A.4.2. LC–MS analysis of the protein deglycosylation

RP-HPLC was carried out C-18 column, (300 x 6 mm, 5 μm Hypersil). The LC separation was carried out with Mobile phase A was made of 0.1% formic acid in Milli-Q water. Mobile phase B was made of 0.1% formic acid in 100% acetonitrile. A linear gradient was run from 0 to 60% B in 30 min (2% B per min). Separation was performed with 35 ml/min flow rate; the column temperature was set at 30°C.
5A.4.3. MALDI-TOF MS experiments

Ultra pure MassPREP™ MALDI matrix, DHB (2,5-Dihydroxybenzoic acid) was used for MALDI-TOF analysis. The matrix was reconstituted in 500 ml of pure ethanol to a final concentration of 20 mg/ml. Purified glycan solutions were mixed with 2,5-dihydroxybenzoic acid (DHB) matrix in one to one ratio; MALDI was used to determine the molecular weight of the released glycans and MS–MS experiments was performed to characterize the structure of the glycans.

5A.5. Results and Discussion

5A.5.1. Influence of denaturation on Deglycosylation:

When deglycosylating a native glycoprotein, it has been recommended that the glycoprotein is subjected to the denaturing protocol. The non-denatured reaction was compared with that of the denatured reaction to determine the extent of reaction completion. It was observed that denaturation was necessary to obtain complete deglycosylation of both OVA and IgY (Figure 5A.1).

Figure 5A.1: Influence of denaturation by SDS-PAGE

Deglycosylation was carried out with both denatured and non-denatured proteins as given under the section (5A.3.1 & 5A.3.2).
Lane: 1 = Control proteins (No denaturation and no deglycosylation).
Lane: 2 = Non-denatured and deglycosylated.
Lane: 3 = Denaturated and deglycosylated.
5A.5.2. Monitoring Deglycosylation

The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS polyacrylamide gels (PAGE). Addition of the enzyme, L-asparaginase resulted in a noticeable increase in mobility of the proteins ovalbumin and IgY (Figure 5A.2). The ability to detect obvious mobility shifts when the sugar is removed will depend on the size of the protein and the relative mass contribution of the sugars removed.

**Figure 5A.2: SDS-PAGE of native and deglycosylated glycoprotein (Ovalbumin and IgY)**

SDS–PAGE was carried out on 10% gel. Lane 1: Molecular Weight Markers; Lane 2: Native IgY; Lane 3: Denatured and deglycosylated IgY. Lane 4: Denatured and deglycosylated ovalbumin; Lane: 5: Native ovalbumin.

The deglycosylated form showed enhanced mobility, presumably due to higher extents of SDS-binding (Walker, 1994). This was in accord with other reports demonstrating a preponderance of carbohydrate moieties in hydrophobic regions of glycoproteins (Wang et al., 1996). A greater affinity of the deglycosylated form for interaction with hydrophobic adsorbents further
supported this observation. Results indicated that upon deglycosylation, flexibility of the enzyme enhanced and its thermostability diminished. Chicken egg albumin, a high mannose protein and egg yolk antibody were used to study deglycosylating properties of L-asparaginase enzyme. The detection of deglycosylating activity was based on the hydrophobicity exhibited by the reactant and the product formed. The product showed higher hydrophobicity due to the loss of the hydrophilic glycan moiety. The difference in the hydrophobicity could be detected by the difference in the retention time on C18 HPLC column, where the hydrophilic compounds eluted earlier than hydrophobic ones. The deglycosylation was based on the difference in hydrophobicity of ovalbumin which was either glycosylated or deglycosylated.

5A.5.3. Effect of temperature in Deglycosylation

Deglycosylation was studied between 16°C through 50 °C using purified L–asparaginase from Cladosporium sp. OVA was incubated with denaturing buffer (5A.2.2.1), pH 7.0 and incubated with L-asparaginase at different temperature for 4 h. Samples were subjected to SDS–PAGE followed by Coomassie staining. It was observed that incubation at 30°C showed complete deglycosylation of OVA (Figure 5A.3).

Figure 5A.3: Influence of temperature on deglycosylation

SDS–PAGE was carried out on 10% gel.
Lane: 1: Control
Lane: 2: 16°C
Lane: 3: 20°C
Lane: 4: 30°C
Lane: 5: 45°C
Lane: 6: 50°C
5A.5.4. Effect of pH in Deglycosylation

Influence of pH on deglycosylation activity was studied between pH values 5.0 through 10.0. Reaction mixture was incubated with denatured OVA in buffer with different pH at 30 °C for 4 h. Samples were subjected to SDS–PAGE followed by Coomassie staining. It was observed that incubation at pH 7.0 showed complete Deglycosylation of OVA (Figure 5A.4).

Figure 5A.4: Influence of pH on Deglycosylation

![SDS-PAGE gel](image)

SDS–PAGE was carried out on 10% gel.
Lane: 1: 5.0
Lane: 2: 6.0
Lane: 3: 7.0
Lane: 4: 8.0
Lane: 5: 9.0
Lane: 6: 10.0

5A.5.5. The LC–MS analysis

The LC–MS analysis was carried out with the electro spray positive ionization (ESI+) coupled with a Micromass Quattro Ultima triple-quadrupole mass spectrometer (Model NO. 2-Tof Ultima, Waters, Manchester, UK). As expected, no signal corresponding to the MW of deglycosylated protein was found in the control sample (Figure 5a.5a). Multiple peaks between 44–45 kDa represented the various N-linked glycoforms of Ovalbumin (Figure 5a.5b). The reaction in the figure 5A.5b shows nearly complete deglycosylation with the protein mass shifted and a prominent peak was detected at approximately 43 kda, which is consistent with the MW of the unmodified protein.
(a) Ovalbumin was solubilised without the use of denaturant and was not deglycosylated. (b) Ovalbumin was denatured and deglycosylated with L-asparaginase. The MS scans were deconvoluted to the MW of the protein. Complete deglycosylation was observed after 4 h.

5A.5.6. MALDI–Tof MS–MS of glycans released from ovalbumin

The MALDI–TOF MS spectra of underivatized N-linked glycans released from 10 pmol Ovalbumin were obtained. MS–MS fragmentations of selected ions were performed to validate the glycan structures. Collision induced dissociation of the complex glycan ion of mass-to-charge ratio of 1948.734 (M 1Na) is given in Figure 5A.6. This ion was observed in the MS mode with low ion intensity however, enough fragmentation ions were produced in the MS–MS mode to determine its structure.
Figure 5A.6: MALDI-Q TOF MS spectrum of the oligosaccharides released from Ovalbumin

MS–MS fragmentation was used to elucidate the structure of glycans.

Analysis of the glycan structure of glycoproteins normally requires enzymatic or chemical methods of Deglycosylation. O-Glycosidic linkages between glycans and the β-hydroxy groups of serine or threonine are readily hydrolyzed by dilute alkaline solutions (0.05 to 0.1 M sodium hydroxide or potassium hydroxide) under mild conditions (45 to 60°C for 8 to 16 hours) leading to the liberation of O-glycans by the mechanism of β-elimination. Hydrolysis is also performed in the presence of a reducing agent (0.8 to 2 M sodium borohydride). This results in the formation of the reduced (alditol) forms of the glycans. N-Linked glycans are not cleaved under these conditions, and neither are O-glycans attached to tyrosine, hydroxyproline, and hydroxylysine. For quantitative release of N-linked glycans, harsher alkaline conditions are required (1 M sodium hydroxide at 100°C for 6 to 12 hours). Again, the reaction must be performed under reducing conditions (1 to 2 M sodium borohydride) to prevent “peeling” reactions taking place on the released N-glycans. N-Acetylglucosamine (GlcNAc) residues are deacetylated during this reaction and must be re-N-acetylated (acetic anhydride in methanol) during the recovery of the glycans.

Chemical Deglycosylation can be done by alkaline β-Elimination and Trifluoromethanesulfonic acid (TFMS) methods but these results in destruction of the glycans. Hence deglycosylation using enzymes are
preferred. The enzyme L-asparaginase brought deglycosylation of ovalbumin and IgY resulting in the subsequent release of free glycans. A complete deglycosylation of proteins was achieved after 4 h incubation with L-asparaginase. Only the enzymatic method has been shown to provide complete sugar removal with no protein degradation.

Removal of carbohydrates from glycoproteins is useful for a number of reasons:
- To simplify analysis of the peptide portion of the glycoprotein.
- To simplify the analysis of the glycan component.
- To remove heterogeneity in glycoproteins for X-ray crystallographic analysis.
- To remove carbohydrate epitopes from antigens.
- To enhance or reduce blood clearance rates of glycoprotein therapeutics.
- To investigate the role of carbohydrates in enzyme activity and solubility.
- To investigate ligand binding.
- For quality control of glycoprotein pharmaceuticals.
5A.6. Summary and conclusions

- L-asparaginase from *Cladosporium* sp. is a deglycosylating enzyme that has been applied in the separation of sugars from protein.
- Denaturation of glycoprotein was observed to be essential for deglycosylation.
- Deglycosylation was observed in both OVA and IgY.
- Deglycosylation was observed at pH 7.0 and temperature 30°C.
- Complete deglycosylation was observed in 4h.
- Deglycosylation products were analysed by SDS-PAGE, HPLC, LC-MS/MS confirmed deglycosylation of OVA and IgY.

5A.7. Bibliography


Chapter 3(B)

Acrylamide reduction in Potato Chips
5B.1. Introduction

Potatoes (*Solanum tuberosum*), one of the world’s major crops, is consumed daily by millions of people from diverse cultural backgrounds (Pedreschi et al., 2004). Findings of AA in some fried and baked foods, most notably potato chips and French fries, at levels of 30-2300 mg/kg by Swedish researchers shocked the food safety authorities. Recently, there has been lot of concern on possible mechanisms of AA formation in foods (Zyzak et al., 2003).

AA is not found in raw potatoes, or formed during boiling. High levels of AA have been reported to be formed at the higher temperatures associated with frying and oven-baking (Ahn et al., 2002). AA formation via Maillard reaction has been confirmed by some international research groups (Coughlin, 2003) and they have illustrated AA formation from food components during heat treatment as a result of the Maillard reaction between amino acids and reducing sugars (Mottram and Wedzicha, 2002). Asparagine, a major amino acid in potatoes and cereals, is responsible for the formation of AA by Maillard reaction at temperatures above 100°C (Friedman, 2003) and asparagine content in potatoes depends on factors like location, fertilization, storage and processing (Davies, 1977; Hippe, 1988). There has been a necessity to mitigate AA formation in heated food products. Thus, the objective of this work was to study and optimise the enzyme concentration, frying temperature, blanching time and time of frying to reduce the level of AA in potato chips using L-asparaginase from *Cladosporium* sp.

5B.2. Materials

5B.2.1. Chemicals

AA (99%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultra-purified water was used throughout the study (Milli-Q Technology, Millipore, Bedford, MA, USA). Syringe filter units (0.45 mm, PVDF) were purchased from Millipore (Bedford, MA) Fine-Chem. Ltd (Mumbai). Potatoes tubers and different oils (Coconut oil, Palm oil, Groundnut oil, Sunflower oil, Ricebran oil, Soyabean oil and Gingly oil) were procured from local market and were stored at 8°C. All other chemicals and solvents used were of analytical grade and were procured from standard chemical companies.
5B.2.2. L-asparaginase enzyme

L-asparaginase enzyme produced from *Cladosporium* sp. on wheat bran by solid state fermentation (3B.4.7) was purified (4A.5.1) and used in the study.

5B.3. Methods

5B.3.1. Preparation of potato slices

Potato tubers were washed and peeled manually. Slices (thickness of 2.0 mm) were cut from the pith of the parenchymatous region of potato tubers using an electric slicing machine (Robot Coupe CL-50, USA). A circular cutting mold was used to provide chips with a diameter of 40 mm.

5B.3.2. Pre-treatments

Potato slices were rinsed immediately for 1 min in dist.\( \text{H}_2\text{O} \) to eliminate some starch material adhering to the surface prior to blanching. Potato slices were then immersed in hot distilled water at 85°C for 3.5 min in a ratio of potato to water (g/g) of \( \simeq 0.5 \) and then sodium metabisulphite (0.15% w/v) was added and kept for another 10 min.

Unblanched chips used in the experiment were only rinsed with dist.\( \text{H}_2\text{O} \) and used for frying.

5B.3.3. Effect of different oils on acrylamide formation in potato chips

The type of oil used for frying was investigated on AA formation in potato chips. Both blanched and unblanched potato slices were deep-fried in different oils separately. Oils used were Coconut oil, Palm oil, Groundnut oil, Sunflower oil, Ricebran oil, Soya bean oil and Gingly oil. Frying was done using an electrical fryer (Dormeyer, model DF3CH, Chicago, USA, 120 V AC, 1100 W), equipped with a static basket and a thermostat. After deep-frying for 10 mins the fried chips were placed on oil absorbent towels. AA concentration was measured in the fried chips.
5B.3.4. Effect of frying time on acrylamide formation in potato chips

Potato slices were prepared as given under (5B.3.1). Both blanched and unblanched potato slices were deep-fried for different time intervals ranging from 3-13 mins. After required time of deep-frying, the potato chips were drained and placed on absorbent towels to remove excess oil. Temperature changes during the deep-frying were measured by two T-type copper-constantan thermocouple probes (Hanna Instruments, Milano, Japan), placed at the sample centre and at 1 mm under the surface of potato samples and temperature was maintained constant. AA concentration was measured in the fried chips.

5B.3.5. Optimization of conditions to reduce acrylamide content in potato chips

A central composite rotatable design (CCRD) with 4 variables was followed to examine the response pattern and to determine the optimum synergy of variables. The parameters namely enzyme concentration (5-305 U), Treatment time (0-40 min), blanching time (0-20 min) and frying temperature (170-190 °C) were optimized to reduce the formation of AA in potato chips. The treatment schedule for CCRD shown in table 5B.2 was arranged to allow for fitting an appropriate regression model using multiple regression program. Six replicates (treatments 27 to 32) at the centre of the design were used for estimation of a pure error sum of squares. The factors were coded at five levels from −2, −1, 0, +1, and +2 as defined by equation (5B.1).

\[
x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1, 2, 3, \ldots k \quad \text{(Eq. 5B.1)}
\]

Where \(x_i\) is the coded value of the variable \(X_i\), \(X_0\) the value of the \(X_i\) at the center point and \(\Delta X_i\) is the increment.

For statistical analysis, the variables \(X_i\) were coded as \(x_i\) according to the transformation Equation (5B.1). The range and levels of the variables in coded units for RSM studies are reported in Table 5B.1. A second-order polynomial equation was used to fit the experimental data given in Table 5B.1. The model proposed for the response (Y) was given by equation (5B.2).
Y = $a_0 + a_1 X_1 + a_2 X_2 + a_3 X_3 + a_4 X_4 + a_{11} X_1 X_2 + a_{22} X_2^2 + a_{33} X_3^2 + a_{44} X_4^2 + a_{12} X_1 X_2 + a_{13} X_1 X_3 + a_{14} X_1 X_4 + a_{23} X_2 X_3 + a_{24} X_2 X_4 + a_{34} X_3 X_4 + \epsilon$ … (Eq.5B.2).

Where $Y$ is the predicted response for AA content (µg/gm) reported in Table 5B.2. $a_0$ is the value of the fitted response at the centre point of the design, $a_i$, $a_{ii}$, $a_{ij}$ being the linear, quadratic, and cross product terms, respectively, and $\epsilon$ is the random error. To deduce workable optimum conditions, a graphical technique was used by fixing two variables at predetermined optimum condition, which was verified by conducting experiments under these conditions (Rastogi et al., 2010). The responses were monitored, and the results were compared with model predictions. The fitted polynomial equation was expressed as surface plots using MATLAB 7.0 to visualize the relationship between the response and experimental levels of each of the factors and to deduce the optimum conditions.

Frying was done using an electrical fryer (Dormeyer, model DF3CH, Chicago, USA, 120 V AC, 1100 W), equipped with a static basket and a thermostat. AA concentration was measured in the fried chips.

**Table 5B.1: Variables and their levels for CCRD**

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<th>Variables</th>
<th>Symbols</th>
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<td>Treatment time (min)</td>
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<td>Blanching time (min)</td>
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<tr>
<td>Frying temp. (°C)</td>
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where $X_1 = (x_1 - 155)/75$; $X_2 = (x_2 - 20)/10$; $X_3 = (x_3 - 10)/5$ and $X_4 = (x_4 - 180)/5$
Table 5B.2: Treatment schedule for 2-factor CCRD and the response

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</table>
5B.4. Analytical

5B.4.1. Extraction of acrylamide

The potato chips were frozen, lyophilized, finely ground and sieved through 40 mesh screen before being used for all the experiments. 1 gm (on dry weight basis) of freeze dried potato chips samples were defatted with 10 ml hexane for 30 mins by leaving the homogenates to stand in water bath set at 30°C under agitation (Tareke et al., 2002). AA from oil free sample was then extracted in water (1:10) by leaving the homogenates to stand in a water bath set at 30°C under agitation. After 30 min the homogenates were centrifuged (12000 rpm, 20 min, 4°C) to allow separation and filtered twice through Whatman No. 2 filter paper. The extracts obtained were filtered through 0.45μm nylon filters and stored refrigerated until analysis.

5B.4.2. Analysis of acrylamide

5B.4.2.1. Analysis by HPLC

A volume of 10 μl of standard and/or sample was injected to Reverse phase C-18 column (300 x 6 mm, 5μm Hypersil), chromatographed in Shimadzu HPLC 10AT series set at isocratic condition with double-distilled water as mobile phase at 1 ml/min flow rate and analyzed at 196 nm (SPD-M10AVP, Shimadzu) at 28 °C (Caulfield et al., 2003). Standard stock solution (1mg/ml) was prepared by dissolving 10 mg of the AA in 10 ml Milli-Q water and stored at 4°C. All working solutions (5-500 µg/L) were freshly prepared by the dilution of stock solution in Milli-Q water. Peak areas of the standards were used to interpolate AA concentrations in the potato ships samples. The retention time of authentic standard AA was 6.2 min under given conditions. All the analyses were performed in triplicate and the average results are expressed as µg/kg sample.

5B.4.2.2. Analysis by LC-MS

HPLC-MS/MS was carried out with the electro spray positive ionization (ESI+) coupled with a Micromass Quattro Ultima triple-quadrupole mass spectrometer (Model NO. 2-Tof Ultima, Waters, Manchester, UK). The mobile phase was 100% methanol followed by 100% water with a flow rate of 0.2ml/min. 2.0 μl of sample was injected to the system. Samples were analyzed in duplicate. Positive ion spectra of the column eluate were recorded.
in the range of \( m/z \) 50–500 at a scan rate of 2.0 s/cycle under the following conditions: capillary voltage, 3.5 kV; cone voltage, 50V; source temperature, 120 °C; desolvation gas temperature, 350 °C; desolvation gas flow, 500 L/h nitrogen; cone gas flow, 50 L/h nitrogen; argon was used as a collision gas at pressure of \( 3 \times 10^{-3} \) mbar for MS/MS, which gave a highest AA response. Data acquisition and processing was performed using MassLynxTM 4.0 SP4 software (Micromass) (Veigas et al., 2007).

5B.4.3. Color determination

Color estimation of untreated and L-asparaginase treated potato chips samples were measured in terms of lightness (L) and color (+ a: red, - a: green, + b: yellow, - b: blue) and total colour difference (ΔE) using CIE Color measuring system (Labscan XE, Hunter Associates Laboratory Inc, Reston, USA). A standard white board made from barium sulphate (100% reflectance) was used as a perfectly white object for setting the instrument with illuminant. Control chips and chips with L-asparaginase were placed in the sample holder and the reflectance was auto-recorded in the wavelength range of 360-800 nm with an observer's angle of 10 °. The total colour change in the \( L^* \), \( a^* \), \( b^* \) colorimetric space, ΔE, was calculated from Equation (5B.31)

\[
\Delta E = \sqrt{((L^*-L_0^*)^2 + (a^*-a_0^*)^2 + (b^*-b_0^*)^2)} \quad \text{Equation (5B.3)}
\]

Where \( L^* \), \( a^* \) and \( b^* \) are the actual colour values and \( L_0^*, a_0^* \) and \( b_0^* \) are the colour values for a control sample, i.e. obtained without L-asparaginase addition. For each run, analyses were made at least in triplicate on three replicated experiments and are expressed as mean ± standard deviation of three readings.

5B.4.4. Texture analysis

Chips firmness (method 74-09) was measured according to AACC (2000) procedure using texture analyzer (Model TA-HDi, Stable Microsystems, Surrey, UK) under the following conditions: sample thickness - 25 mm, load cell – 10 Kg, plunger diameter – 36 mm and plunger speed – 100 mm per minute. Chips firmness, in terms of force (g) required for 25 % compression was measured.
5B.4.5. Sensory Analysis of Potato chips

The optimised conditions obtained from RSM were used to prepare enzyme treated potato chips and control chips. The chips prepared were used for evaluation of taste, texture, appearance, color, odor and overall acceptability (Carpenter et al., 2000). Three samples of potato chips were taken for Sensory analysis. They were coded as i. S₁ (unblanched), ii. S₂ (blanched) and iii. S₃ (Blanched and enzyme treated). Sensory profiling and consumer acceptance study were carried out for these three set of samples.

5B.4.5.1. Sensory Profiling

Sensory profiling of potato chips was carried out by a trained panel in sensory booths. Evaluations were conducted under white fluorescent light, with the booth area maintained at temperature 22± 2°C and RH 50± 5%.

5B.5.5.2. Sample preparation

The samples were bland. About 2-3 pieces of potato chips were served in small porcelain plates coded with three digit random numbers. A glass of warm water was also served to cleanse the palate.

5B.5.5.3. Sensory analysis

Quantitative Descriptive Analysis (QDA) method was employed for profiling the samples. A suitable score card comprising selected sensory attributes (descriptors) was formulated for this purpose. Panellists were asked to mark on a scale of 0-15 cm to indicate the perceived intensity of each attribute listed on the score card. The scale was anchored at 1.25 cm on either end, representing ‘Recognition Threshold’ and ‘Saturation Threshold’ respectively. Scores given for all the attributes for each sample were tabulated. Next, mean value was calculated for each attribute of a sample, representing the panel’s judgment about the sensory quality of the product. These are depicted graphically as ‘Sensory Profiles’.
5B.5. Results and Discussion

5B.5.1. Effect of blanching and different oils on the formation of AA in potato Chips

Frying time of 7 min was optimized to get crispy chips. Table 5B.3 shows the effect of different oils on AA formation in potato chips with and without blanching pre-treatments. Frying using coconut oil showed increase in formation of AA by 13 min of frying. The concentration of AA formed increased with time. With blanching the formation of AA was 3.4 times less compared with samples without blanching. Palm oil frying of potato chips resulted in very low AA formation compared with other oils. Here also blanching helped in reduction of AA by 4 folds, i.e., in samples without blanching 223 µg of AA was formed which got reduced to 51 µg due to blanching. Frying with groundnut oil had 2 times more AA formation even with blanched potatoes i.e., the AA concentration was 311 µg compared to 684 µg obtained without blanching by 13 min of frying. Un-blanched potato chips had 4 times more AA compared in palm oil fried blanched chips i.e., the blanched chips had 51 µg of AA. When sunflower oil was used as a frying medium, the AA formed in blanched potatoes was 1.25 times less than in unblanched potato chips. The concentration of AA formed with this oil was 180 and 121 µg even at low frying time of 3 min respectively with unblanched and blanched potato chips. With rice bran oil as frying medium, the AA formed was lowest next to palm oil. Gingly oil medium resulted in highest AA formation both with and without blanching. Frying with soyabean oil resulted in AA formation which was second highest among the oils used.

It was observed that AA formation increased with frying time in all the frying media used. Highest level of 981 µg of AA reached in gingly oil by 13 mins of frying, it was nearly 3 times than in 3 min frying. In soyabean oil, the increase in AA level (808 µg) was 9.3 times more than 3 min frying. In both rice
Table 5B.3: Effect of different oils and frying time on acrylamide formation in potato chips

<table>
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<tr>
<th>Sl No.</th>
<th>Oil Type</th>
<th>Time of frying (min)</th>
<th>Acrylamide Content (µg/gm)</th>
<th>Without blanching</th>
<th>With blanching</th>
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<td>186.28</td>
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<td>13</td>
<td>808.22</td>
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<td>361.58</td>
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<td>13</td>
<td>981.28</td>
<td>680.28</td>
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bran oil and palm oil no AA was formed at 3 h of frying. The results indicated that palm oil mediated low AA formation and gingly oil mediated highest AA formation. Thus type of oil used for frying had a significant role in AA formation in a potato chips model system. Some studies indicated that palm oil relatively
to rape seed and sunflower oils generated higher AA contents (Gertz and Klostermann, 2002) and olive oil in comparison to corn oil in French fries model (Becalski et al., 2003). Blanching helped in reducing AA formation with all kinds of oil media tested for frying. This could be due to the leaching out of reducing sugars, prior to frying, inhibiting the non-enzymatic browning reactions and leading to reduction in AA formation. Similar observations have been made by Pedreschi and Moyano, (2005) with French fries which were lighter and less red. Drying of blanched or soaked potato slices prior to final frying reduces fat absorption and oil hydrolysis (Choe and Min, 2007). In addition, it also reduces AA formation in French fries due to the need of shorter finish frying times to obtain the same product quality in terms of color and crispiness (Franke et al., 2005).

5B.5.2. Optimization and validation of the experimental model

The coefficients of Equation 5B.1 were obtained using MATLAB 7.0 software (The MathWorks Inc., Natick, MA, USA) based on the data provided in Table 5B.2 and are presented in Table 5B.4. The ‘t’ values of the estimate were compared with the tabular value, and the terms having ‘t’ values lower than the tabular values were omitted (Khuri and Cornell, 1987). All the four responses under different combinations as defined in the design (Table 5B.1) were analyzed using the analysis of variance appropriate to the experimental design (Table 5B.5), which indicated that the sum of squares because of regression (first- and second-order terms) was significant. However, the high values of coefficient of determination ($R^2$, Table 5B.5) also suggest that the model is a good fit. The $R^2$ is the proportion of variability in response values explained or accounted for by the model (Myers, 1971, Montgomery, 1984).

The effect and responses of enzyme-concentration (5-305 U), Treatment time (0-40 min), blanching time (0-20 min) and Frying temperature (170- 190 °C) on AA formation are presented by the coefficients of second-order polynomials (Table 5B.4). Response surfaces based on these coefficients are also shown in Figure 5B.1. The response surfaces were selected based on the observation of the data and initial optimization of the individual responses. In general, exploration of the response surfaces indicated a complex interaction between the variables.
### Table 5B.4: Estimated coefficient for the fitted second order polynomial representing the relationship between the response and process variables

<table>
<thead>
<tr>
<th>Acrylamide content (µg/gm)</th>
<th>Estimated coefficients</th>
<th>t-value</th>
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<tr>
<td>a₀</td>
<td>5.79 (^a)</td>
<td>7.73</td>
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<tr>
<td>a₁</td>
<td>-3.34 (^a)</td>
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<td>a₂</td>
<td>-1.48 (^b)</td>
<td>3.67</td>
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<td>a₃</td>
<td>-1.11 (^b)</td>
<td>2.74</td>
</tr>
<tr>
<td>a₄</td>
<td>0.75 (^c)</td>
<td>1.84</td>
</tr>
<tr>
<td>a₁₁</td>
<td>2.64 (^a)</td>
<td>7.13</td>
</tr>
<tr>
<td>a₂₂</td>
<td>-0.71 (^c)</td>
<td>1.92</td>
</tr>
<tr>
<td>a₃₃</td>
<td>-0.60 (^{ns})</td>
<td>1.61</td>
</tr>
<tr>
<td>a₄₄</td>
<td>-0.16 (^{ns})</td>
<td>0.44</td>
</tr>
<tr>
<td>a₁₂</td>
<td>-0.51 (^{ns})</td>
<td>1.02</td>
</tr>
<tr>
<td>a₁₃</td>
<td>0.46 (^{ns})</td>
<td>0.93</td>
</tr>
<tr>
<td>a₁₄</td>
<td>-0.61 (^{ns})</td>
<td>1.22</td>
</tr>
<tr>
<td>a₂₃</td>
<td>1.41 (^b)</td>
<td>2.84</td>
</tr>
<tr>
<td>a₂₄</td>
<td>0.67 (^{ns})</td>
<td>1.35</td>
</tr>
<tr>
<td>a₃₄</td>
<td>0.19 (^{ns})</td>
<td>0.38</td>
</tr>
</tbody>
</table>

\(^a\) Significant at 0.1\%, \(^b\) Significant at 1.0\%, \(^c\) Significant at 5.0\%

\(^{ns}\) Not significant even at 5% level
Table 5B.5: Analysis of variance for the fitted second order polynomial model and lack of fit for biomass yield as per CCRD

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Sum of squares</th>
<th>Mean sum of squares</th>
<th>F value</th>
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</thead>
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<tr>
<td>Regression</td>
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</tr>
<tr>
<td>First order terms</td>
<td>4</td>
<td>362.63**</td>
<td>90.66</td>
<td>1951.83</td>
</tr>
<tr>
<td>Second order terms</td>
<td>10</td>
<td>300.96**</td>
<td>30.10</td>
<td>647.96</td>
</tr>
<tr>
<td>Total</td>
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<td>663.59</td>
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<td></td>
</tr>
<tr>
<td>Residual</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>11</td>
<td>62.49 ns</td>
<td>5.68</td>
<td>122.31</td>
</tr>
<tr>
<td>Pure error</td>
<td>6</td>
<td>0.28</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Total error</td>
<td>17</td>
<td>62.77</td>
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<td></td>
</tr>
<tr>
<td>Grand Total</td>
<td>31</td>
<td>726.36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Coefficient of Determination ($R^2$) = 0.914

*Significant at 5% level, ** Significant at 1% level, ns Not significant

5B.5.2.1. Effect of treatment time and enzyme concentration on AA reduction

At lower enzyme concentration of 5 U the AA concentration was 15 µg/gm potato chips. As the enzyme concentration was increased to 200 U the AA concentration reached the lowest i.e 2 µg/g. Further increase in enzyme concentration did not affect further reduction in AA concentration. The enzymatic lowering of AA formation was time dependent. Maximum reduction in AA formation reached by 20 min of enzyme treatment (Figure 5B.1a).

5B.5.2.2. Effect of blanching time and enzyme concentration on AA reduction

The blanching time also played an important role in reducing AA. However at low enzyme concentrations and increased blanching time, reduction in AA was not significant. At low enzyme concentration of 5 U the AA concentration was 13 µg/g. At enzyme concentration of 305 U the AA concentration was 6 µg/g (Figure 5B.1b).
Figure 5B.1: Surface plots for interaction
(a) treatment time and enzyme concentration; (b) blanching time and enzyme concentration; (c) frying temperature and enzyme concentration; (d) blanching time and treatment time; (e) frying temperature and treatment time; (f) frying temperature and blanching time. Levels are as shown in Table 5B.1.

5B.5.2.3. Effect of frying temperature and enzyme concentration on AA reduction

The interaction between frying temperature and enzyme concentration are given in Figure 5B.1c. At high frying temperature, the AA formation was high when enzyme was present at low concentrations. With decrease in frying time and increase in enzyme concentration, the AA concentration decreased.

5B.5.2.4. Effect of blanching time and treatment time on AA reduction

The treatment time also had a significant influence on AA formation. At low blanching time and low enzyme treatment time the AA formation was high, it was around 10 μg/g of potato chips. Increase in blanching time i.e., 15 min and enzyme treatment time of 30 mins significantly reduced AA formation (Figure 5B.1d).
5B.5.2.5. Effect of frying temperature and treatment time on AA reduction

The interactions between frying temperature and treatment time are given in Figure 5B.1e. Even though the frying temperature was low, there was sufficient AA (10 µg/g) formation at low enzyme treatment time. With increase in treatment time the AA concentration was only 3 µg/g even at 190°C.

5B.5.2.6. Effect of frying temperature and blanching time on AA reduction

Blanching time of 20 min helped in maximum reduction of AA even at 190°C frying temperature. Figure 5B.1f shows the frying temperature and blanching time on AA formation. At low frying temperature the AA formed was insignificant and with less blanching, there was AA formation.

5B.5.3. Validation of results

The suitability of the model equations for predicting the optimum response values was tested using recommended optimum conditions, determined by response surface methodology optimization approach, which validated experimental and predicted values of the responses using the model equations. The conditions for AA reduction are as shown in the Table 5B.6. With 10 min of blanching and treatment time of 36 min with 200 U of L-asparaginase reduced acrylamide to 94 % after frying at 180°C. These optimized conditions could be considered as optimum as well as feasible conditions. These set of conditions were determined to be optimum by the response surface methodology optimization approach and were also used to validate experimentally and the predicted values using model equation (Equation 5B.1). The experimental values were found to be in agreement with the predicted values (Table 5B.6).
Table 5B.6: Feasible optimum conditions and predicted and experimental value of response at optimum condition

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Enzyme Conc. (Unit)</th>
<th>Treatment time (min)</th>
<th>Blanching time (min)</th>
<th>Frying temperature (°C)</th>
<th>Acrylamide content (µg/gm) (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>200.54 (0.61)</td>
<td>36.12</td>
<td>10.00</td>
<td>180.00</td>
<td>0.00 (0.0±0.00)</td>
</tr>
<tr>
<td>B</td>
<td>229.09 (0.99)</td>
<td>36.17</td>
<td>10.00</td>
<td>180.00</td>
<td>0.00 (0.0±0.00)</td>
</tr>
<tr>
<td>C</td>
<td>242.65 (1.17)</td>
<td>36.80</td>
<td>10.00</td>
<td>180.00</td>
<td>0.00 (0.0±0.00)</td>
</tr>
<tr>
<td>D</td>
<td>278.92 (1.65)</td>
<td>40.00</td>
<td>10.00</td>
<td>180.00</td>
<td>0.00 (0.0±0.00)</td>
</tr>
<tr>
<td>E</td>
<td>211.25 (0.75)</td>
<td>35.92</td>
<td>10.00</td>
<td>180.00</td>
<td>0.00 (0.0±0.00)</td>
</tr>
</tbody>
</table>

The values in the parenthesis for independent variables are the coded values. For the response, the values in the parenthesis are the experimental values representing the mean and standard deviation of five experiments. The condition A has been selected as optimum condition.

5B.5.4. Confirmation of acrylamide in potato chips

The acrylamide positive samples, showing peaks in HPLC were analysed by LC-MS-MS to confirm the presence of acrylamide. All the HPLC positive samples were found to be positive with MS analysis with a molecular mass of 70 (Figure 5B.2a & 2b).

Figure 5B.2a: LC-MS pattern of Standard acrylamide
Chapter 3B  Acrylamide reduction in Potato Chips

Figure 5B.2b: LC-MS pattern of food sample (potato chips) containing acrylamide

5B.5.5. Sensory evaluation of L-asparaginase treated potato chips

5B.5.5.1. Colour evaluation of potato crisps

The surface colour was measured by CIE Color measuring system (Hunter colorimeter). The results, presented in Table 5B.7, are expressed as the difference in colour compared to raw potato tissue (ΔE). Accordingly, a higher ΔE value corresponded to a darker and browner product. From the colour measurements, it could thus be concluded that the blanching and enzyme treatment produced chips same as control chips, with less amount of AA. Chips with L-asparaginase enzyme showed crust colour with slight lower L* value than control chips without L-asparaginase enzyme (Table 5B.7). However the color of the chips of both control and enzyme treated were uniform (Figure 5B.3a & b). The redness (a*) and yellowness (b*) of the chips was also not significantly different between all samples (Table 5B.7). There was no correlation found between browning (ΔE) and the AA content in potato crisps. Previously, it was also shown that addition of NaCl and CaCl₂ to potato strips did not change the surface colour upon subsequent frying (Bunger et al., 2003; Franke et al., 2005; Gokmen and Senyuva, 2007), although other studies found a reduced browning upon addition of NaCl (Pedreschi et al., 2007a; Pedreschi et al., 2007b). The effect of L-asparaginase treatment on the color of potato chips was tested by Pedreschi et al.(2011) and reported that values of L* a* and b* components did not differ considerably among all the pre-treatments. Addition of the enzyme did not affect the color (P>0.05), in agreement with literature report (Capuano et al., 2009; Kukurova et al., 2009).
Figure 5B.3a: Photograph of control/untreated potato chips

![Control/untreated potato chips]

Figure 5B.3b: Photograph of chips treated with L-asparaginase

![Chips treated with L-asparaginase]
Table 5B.7: Colour analysis of Potato chips

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>ΔE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34.01</td>
<td>7.02</td>
<td>11.83</td>
<td>56.23</td>
</tr>
<tr>
<td>2</td>
<td>35.65</td>
<td>7.49</td>
<td>13.62</td>
<td>57.16</td>
</tr>
<tr>
<td>3</td>
<td>35.14</td>
<td>6.69</td>
<td>15.78</td>
<td>56.33</td>
</tr>
<tr>
<td>4</td>
<td>35.85</td>
<td>6.77</td>
<td>11.74</td>
<td>58.59</td>
</tr>
<tr>
<td>5</td>
<td>36.34</td>
<td>5.48</td>
<td>13.52</td>
<td>55.08</td>
</tr>
<tr>
<td>6</td>
<td>34.28</td>
<td>7.41</td>
<td>12.49</td>
<td>54.53</td>
</tr>
<tr>
<td>7</td>
<td>35.23</td>
<td>6.27</td>
<td>12.58</td>
<td>55.92</td>
</tr>
<tr>
<td>8</td>
<td>36.28</td>
<td>7.51</td>
<td>12.03</td>
<td>56.29</td>
</tr>
<tr>
<td>9</td>
<td>36.79</td>
<td>6.21</td>
<td>13.35</td>
<td>54.31</td>
</tr>
<tr>
<td>10</td>
<td>35.62</td>
<td>6.29</td>
<td>13.89</td>
<td>55.81</td>
</tr>
<tr>
<td>11</td>
<td>33.51</td>
<td>6.57</td>
<td>13.84</td>
<td>54.79</td>
</tr>
<tr>
<td>12</td>
<td>35.78</td>
<td>7.24</td>
<td>13.56</td>
<td>55.06</td>
</tr>
<tr>
<td>13</td>
<td>34.01</td>
<td>7.21</td>
<td>14.61</td>
<td>57.23</td>
</tr>
<tr>
<td>14</td>
<td>33.96</td>
<td>7.32</td>
<td>12.14</td>
<td>56.19</td>
</tr>
<tr>
<td>15</td>
<td>33.72</td>
<td>7.24</td>
<td>11.81</td>
<td>56.62</td>
</tr>
<tr>
<td>16</td>
<td>35.41</td>
<td>7.12</td>
<td>13.17</td>
<td>56.59</td>
</tr>
<tr>
<td>17</td>
<td>34.54</td>
<td>7.32</td>
<td>12.27</td>
<td>57.35</td>
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<td>7.02</td>
<td>13.29</td>
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<td>19</td>
<td>33.77</td>
<td>6.93</td>
<td>11.62</td>
<td>57.75</td>
</tr>
<tr>
<td>20</td>
<td>32.72</td>
<td>6.55</td>
<td>13.65</td>
<td>56.36</td>
</tr>
<tr>
<td>21</td>
<td>33.21</td>
<td>6.71</td>
<td>13.71</td>
<td>55.75</td>
</tr>
<tr>
<td>22</td>
<td>34.09</td>
<td>6.91</td>
<td>12.03</td>
<td>55.99</td>
</tr>
<tr>
<td>23</td>
<td>33.18</td>
<td>6.12</td>
<td>10.44</td>
<td>55.83</td>
</tr>
<tr>
<td>24</td>
<td>36.98</td>
<td>6.16</td>
<td>14.97</td>
<td>54.48</td>
</tr>
<tr>
<td>25</td>
<td>33.14</td>
<td>6.95</td>
<td>13.93</td>
<td>55.81</td>
</tr>
<tr>
<td>26</td>
<td>33.86</td>
<td>6.08</td>
<td>13.88</td>
<td>54.67</td>
</tr>
<tr>
<td>27</td>
<td>32.73</td>
<td>6.75</td>
<td>13.54</td>
<td>56.76</td>
</tr>
<tr>
<td>28</td>
<td>33.28</td>
<td>6.91</td>
<td>15.52</td>
<td>54.73</td>
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<td>34.52</td>
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</tr>
<tr>
<td>30</td>
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</tr>
<tr>
<td>31</td>
<td>35.25</td>
<td>6.89</td>
<td>13.52</td>
<td>55.62</td>
</tr>
</tbody>
</table>

(Serial no as per treatment schedule of CCRD)
Data represent the mean of three replicates ± standard error.
L* = lightness, higher values indicate lighter colour.
a* = redness.
b* = yellowness; higher colour intensity is indicated by higher values.
5B.5.5.2. Sensory analysis of potato chips

Preliminary sensory evaluation revealed that there was no difference in aroma or taste of these samples. There was no off flavour or off taste such as bitterness perceived in these samples. Once this was confirmed, rest of the potato chips was coated with 0.5 % salt and 0.25 % freshly powdered pepper to simulate market samples. Visual examination of potato chips revealed that $S_1$ was slightly dull in color and had brown color along the edges while $S_2$ and $S_3$ were lighter and uniform in color and had better visual appeal. Results of sensory analysis indicated that $S_2$ and $S_3$ were perceived to have high crispness (8.4 and 8.0 respectively) and crunchiness (8.8 and 9.1 respectively). Sample $S_3$ had higher perceived intensity of fried potato aroma closely followed by that in $S_2$ which was significantly higher to that in $S_1$. Overall quality of $S_2$ and $S_3$ was rated at 9.8 and 10 respectively which were significantly higher than that of $S_1$ (Figure 5B.4). It was observed that $S_2$ and $S_3$ had a clean taste and mouthfeel while $S_1$ had a mild ‘fried starchy’ aftertaste sensed with retronasal perception.

Figure 5B.4: Sensory profile of potato chips

S$1$-unblanched; S$2$ - blanched and S$3$- Blanched and enzyme treated.
5B.5.5.3. Consumer acceptance study

Consumer acceptance study on potato chip samples was carried out to assess their acceptability by potential consumers. The product was rated for ‘Color’, ‘Texture’ and ‘Overall Quality’. Respondents who participated in this study comprised of staff and students (both sex) from various departments at CFTRI. Their age ranged between 22 and 55 years. This segment of population included people from different regions of India and different socio-economic strata. A seven-point Hedonic scale ranging from “Like Very Much” to “Dislike Very Much” with “Neither Like nor Dislike” as the midpoint was used for this test. Results showed that majority of respondents rated the products S₂ and S₃ mostly on ‘Like’ side of the scale. Interestingly, there was no marking on the negative side/dislike side of the scale for the color of S₂ and S₃ whereas 22% of respondents disliked the color of S₁ (Figure 5B.5a). More than 85% of respondents rated texture of S₂ and S₃ on the ‘Like’ side/positive side of the scale whereas only 50% of respondents rated texture of S₁ on the ‘Like’ side. When overall quality was considered, only 2.8% and 33.3% of respondents rated S₁ “like very much’ and ‘Like moderately’ respectively. However, for S₂, it was over 47% and 33% respectively for these same levels of liking. In other words, ratings for S₂ (Figure 5B.5b) showed a clear pattern of ‘Like’ by about 80% of respondents. Similar was the case with S₃ (Figure 5B.5c) where over 47% of respondents rated it ‘Like very much’ and 27% rated it ‘Like moderately’ and over 19% rated it ‘Like slightly’. This showed that compared to S₁, samples S₂ and S₃ were liked to a greater extent by over 80% of respondents indicating their high degree of acceptability. There was no significant impact on the flavour, color, and other sensory properties on treatment with L-asparaginase. The deep-fried potato samples subjected or not to the dipping treatments resulted not significantly different for flavour, sourness and crispness (Anese et al., 2009). The application of commercial L-asparaginase for elimination of one of the main precursors, amino acid L-asparagine without compromising the sensory properties of processed products was reported by Ciesarova et al. (2006).
Chapter 3B  
Acrylamide reduction in Potato Chips

Figure 5B.5a: Consumer acceptance Test - Sample S₁

Sample S₁ – unblanched potato slices and then deep-fried.

Figure 5B.5b: Consumer acceptance Test - Sample S₂

Sample S₂ – blanched potato slices and then deep-fried.
Figure 5B.5c: Consumer acceptance Test - Sample S₃

Sample S₃ - Blanched, enzyme treated potato slices and then deep-fried.
5B.6. Summary and Conclusions

- L-asparaginase mediated reduction in acrylamide formation in potato chips was optimized.
- Among frying oils tested palm oil was found to be the best yielding very low acrylamide.
- Blanching helped in reducing acrylamide formation.
- Enzyme treatment coupled with blanching reduced acrylamide formation in potato chips significantly.
- 94% reduction in acrylamide formation in potato chips fried at 180°C was observed with 10 min of blanching and treatment time of 36 min with 200 U of L-asparaginase.
- Sensory evaluation studies indicated the L-asparaginase treated product to be better than control chips.
- L-asparaginase treated chips were highly acceptable than control chips.
- An integrated approach is thus required, which combines the knowledge how to reduce AA with the corresponding product-specific quality aspects, such as colour, texture and taste.
- The use of additives might also stimulate other reactions upon frying, which may negatively influence product safety.
- These studies open avenues for the application of microbial enzymes in obtaining safe foods without compromising the organoleptic properties.
5B.7. Bibliography


Coughlin JR. Acrylamide: What we have learned so far. *Food Technology.* 2003; 57(2): 100-104.


Gokmen V & Senyuva HZ. Acrylamide formation is prevented by divalent cations during the Maillard reaction. *Food Chemistry*, 2007; 103: 96-203.


Chapter 3(C)

**Acrylamide reduction in Sweet bread**
5C.1. Introduction

Dough based foods are commonly used for breakfast and snacks like breads and biscuits. Bakery products are the most important type of processed food worldwide and of these the most consumed product is bread. These are rich in carbohydrates and other sugars and usually are baked at high temperatures around 180°C to 220°C to get proper texture and taste. At this high temperatures the sugars present in the flour will get caramelized and undergo Maillard reaction. Maillard reaction is important for the formation of colour and aroma in the bread crust, but may also be associated with the formation of toxic compounds, such as acrylamide (AA) and 5-hydroxymethylfurfural (HMF) (Mottram et al., 2002). Haase et al. (2003) discussed the formation of AA in baked products, and identified flour milling intensity and baking temperature as important factors affecting AA concentration in bread. Thus studies explaining the formation of AA and providing measures that control its reduction in foods are imperative. AA is reported to be a probable human carcinogen (Hogervorst et al., 2007; IARC, 1994; Olesen et al., 2008). Considerable attempts have been made to mitigate the formation of the potential hazardous Maillard reaction products (MRPs) in thermally processed foods with special attention to AA either by selection of suitable raw material (Claus et al., 2006b; De Wilde et al., 2006), changes in the formulation, or by optimization of the process technology. The aim of the present study was to investigate the effects of L-asparaginase on the rheological characteristics of wheat flour, physico-sensory, chemical characteristics of bread and AA formation during baking of sweet bread. This knowledge is important since it provides alternatives that improve reduction of AA in bread.

5C.2. Materials

5C.2.1. Chemicals

AA (>99%), HMF and L-asparagine were purchased from Sigma–Aldrich chemical company. Potassium ferrocyanide, Zinc acetate, potassium phosphate, Bromo-cresol green, methyl red and Boric acid were procured from Hi Media Laboratories Ltd., Mumbai, India. All other chemicals and solvents used were of analytical grade and were procured from standard
chemical companies. Ultra pure water (Milli-Q system, USA) was used throughout the experiments. High performance liquid chromatographic (HPLC) grade solvents Methanol and acetonitrile were obtained from Qualigens Fine Chemicals, Glaxo (India) Ltd., Mumbai and E. Merck (India) Ltd., Mumbai. Compressed yeast (Tower brand, AB Mauri, India Pvt Ltd., Chennai, India), hydrogenated fat (Bunge India Pvt Ltd., Mumbai, India); salt (common food grade sodium chloride).

5C.2.2. L-asparaginase enzyme

L-asparaginase enzyme produced from Cladosporium sp. on wheat bran by solid state fermentation (3B.4.7) was purified (4A.5.1) and used in the study.

5C.2.3. Wheat flour and bread ingredients

Commercial refined wheat flour having 11.6% moisture, 0.5% ash, 10.1% protein, 26 ml Zeleny’s sedimentation value and 586 sec Hagberg falling number was used for the study. Ingredients like compressed yeast, hydrogenated fat, water, salt and sugar were also used.

5C.2.4. Reagents

5C.2.4.1. HMF analysis

- **Potassium ferrocyanide (15%)**

<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium ferrocyanide</td>
<td>: 150 g</td>
</tr>
<tr>
<td>dist.H$_2$O</td>
<td>:1000 ml</td>
</tr>
</tbody>
</table>

- **Zinc acetate (30%)**

<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc acetate</td>
<td>: 300 g</td>
</tr>
<tr>
<td>dist.H$_2$O</td>
<td>:1000 ml</td>
</tr>
</tbody>
</table>

- **Acetonitrile (5%)**

<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>: 5 ml</td>
</tr>
<tr>
<td>dist.H$_2$O</td>
<td>: 95 ml</td>
</tr>
</tbody>
</table>
5C.2.4.2. L-asparagine analysis

- **Tri-chloro acetic acid (5%)**
  
  - Tri-chloro acetic acid: 5 ml
dist.\text{H}_2\text{O}: 95 ml

- **Potassium phosphate (0.03M)**
  
  - Potassium phosphate: 4.08 g/L
dist.\text{H}_2\text{O}: 95 ml

  pH was adjusted to 3.2 by adding concentrated orthophosphoric acid.

5C.2.4.3. Total sugar estimation

- **Standard sugar solution:**
  
<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>2 g</td>
</tr>
<tr>
<td>dist.\text{H}_2\text{O}</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

- **Methylene blue indicator**
  
<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene blue</td>
<td>2 g</td>
</tr>
<tr>
<td>dist.\text{H}_2\text{O}</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

- **Fehling’s solution:** Equal volumes of solution A and solution B prepared as described below. (Prepared immediately before use).

  **Solution A:**
  
  \[
  \text{CuSO}_4.5\text{H}_2\text{O} : 34.63 \text{ g} \\
  \text{H}_2\text{SO}_4 (\text{sp. Gr. 1.83}) : 0.5 \text{ ml} \\
  \text{dist.}\text{H}_2\text{O} : 500 \text{ ml}
  \]

  Dissolve 34.639 g of copper sulphate (\text{CuSO}_4.5\text{H}_2\text{O}) in water add 0.5 ml of concentrated sulphuric acid and diluted to 500 ml in a volumetric flask. Solution was filtered through asbestos.

  **Solution B:**
  
<table>
<thead>
<tr>
<th>Components</th>
<th>Grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium sodium tartarate (Rochelle salt)</td>
<td>173 g</td>
</tr>
<tr>
<td>\text{NaOH}</td>
<td>50 g</td>
</tr>
<tr>
<td>dist.\text{H}_2\text{O}</td>
<td>500 ml</td>
</tr>
</tbody>
</table>
Dissolve 173 grams of Rochelle salt (potassium sodium tartarate-KNaC$_4$H$_4$O$_6$.4H$_2$O) and 50 grams of sodium hydroxide in water, diluted to 500 ml in volumetric flask and the solution was allowed to stand for two days. Solution was filtered through asbestos.

- **Zinc acetate solution**
  Crystalline zinc acetate : 21.9 g
  glacial acetic acid : 3 ml
  dist.H$_2$O : 100 ml

- **Potassium ferrocyanide solution**
  Potassium ferrocyanide : 10.6 g
  dist.H$_2$O : 100 ml

- Concentrated Hydrochloric acid solution (Specific Gravity: 1.17).

- Concentrated ammonia solution (Specific Gravity: 0.88).

- **Ammonia solution**
  Ammonia solution : 10 ml
  dist.H$_2$O : 90 ml.

### 5C.2.4.4. Protein estimation

- **Digestion Mixture**: Powdered potassium sulphate, copper sulphate and selenium dioxide were mixed thoroughly in the ratio 5:2:1.

- **Sodium hydroxide solution (40%)**
  NaOH : 40 g
  dist.H$_2$O : 100 ml

- **Boric acid (2%)**
  Boric acid : 2 g
  dist.H$_2$O : 100 ml.

- **Mixed indicator**:
  Bromo-cresol green : 0.1 g
  Methyl-red : 0.1 g
  Absolute alcohol (Ethanol) : 100 ml
0.1% solution each of Bromo-cresol green and methyl red were prepared separately in absolute alcohol and then 5 parts of the Bromo-cresol green was mixed with one part of methyl red.

- **N/70 hydrochloric acid**: Approximately 1.3 cc of Hydrochloric acid was dissolved in 1 L of water.

- **Ammonium sulphate**:

<table>
<thead>
<tr>
<th>Components</th>
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</thead>
<tbody>
<tr>
<td>Ammonium sulphate</td>
<td>0.942</td>
</tr>
<tr>
<td>dist. H₂O</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

  5 ml of ammonium sulphate solution as equivalent to 1 mg of nitrogen.

**5C.2.4.5. DNS (3, 5-Dinitrosalicylic acid) reagent**

**Solution 1**: Sodium Potassium tartrate : 135 g
- dist. H₂O : 225 ml

**Solution 2**: 3.5-dinitrosalicylic acid : 4.5 g
- Sodium Hydroxide (NaOH) : 7.2 g
- dist. H₂O : 90 ml

Solution 1 and solution 2 are mixed in 1 L conical flask and was kept in boiling water bath to get a clear homogenate. The volume was made upto 450 ml by adding dist. H₂O.

**5C.3. Methods**

**5C.3.1. Zeleny’s sedimentation value**

3.2 g wheat flour was placed in 100 ml stoppered measuring cylinder containing 50 ml of water with Bromophenol blue. Mixture was thoroughly mixed using shaker for 5 min. At the end of 5 min, 25 ml of isopropyl alcohol was added and then again kept on shaker for 5 min. The cylinder was kept in the upright position and at the end of exactly 5 min, volume of sediment in ml was recorded. This represents directly sedimentation value in ml, which is then expressed on 14% moisture basis according AACC (method 56-61).

**5C.3.2. Falling number**

7 g wheat flour was weighed and placed in the falling number tube. 25 ml of water was added. Rubber stopper was inserted and the tube was
shaken for 20 times such that the entire flour was suspended in water. Stirrer was placed in the tube and the tube was placed in water bath (Falling Number apparatus). Automatic stirring of flour suspension was carried out for 1 min. The falling was recorded in terms of time with the stirrer falling a fixed distance through the liquefied gel. Falling number was reported based on 14% moisture. (AACC method 56-81B).

5C.3.3. Rheological characteristics

Effect of 50, 100, 200 and 300U L- asparaginase enzyme on the rheological characteristics of wheat flour, such as farinograph (method 54-21) and amylograph (method 22-10) were determined individually using AACC (2000) methods.

5C.3.3.1. Farinograph characteristics of wheat flour

Brabender Farinograph is the universally used physical dough testing equipment by means of which water absorption and mixing profile of the dough can be determined. 50g of wheat flour (14% moisture) was weighed and taken in mixer bowl. 1 minute of dry mixing at high speed was done to homogenize the flour. Water was added through burette (say 30ml initially) and mixed. Further, the quantity of water can be adjusted, so that maximum consistency of 500 BU is attained. Water should be added at a stretch and farinograph is allowed to run for 10 min. Farinograph characteristics (Figure 5C.1) were measured as below;

a) Water absorption of the flour on 14% moisture basis using equation 5C.1:

Absorption % = 2 \times (x + y - 50) \quad \text{(Eq. 5C.1)}

Where: \( x = \text{ml of water added} \); \( y = \text{gm of flour used} \).

Percentage of water within dough at a consistency such that the Farinograph curve centers 500 BU line at point of maximum development.

b) Dough development time:

Time in minutes from start of mixing to attainment of peak.

c) Stability:

Time in minutes for which the graph remains at 500 BU line.


d) Mixing Tolerance Index:
The drop in Brabender units (BU) after 5 min from the peak.

Figure 5C.1: Specimen curve illustrating the interpretation of the farinogram

5C.3.3.2. Micro-visco amylograph characteristics of starch and wheat flour

Micro visco-amylograph is an instrument used for measuring the gelatinization properties of starch and flour. The instrument is equipped with an integrated, self-optimizing temperature control unit together with windows software.

Method: The conditions of the test are as follows: Sample weight - 15 g, water addition - 100 ml, heating rate - 7.5°C/min, measuring range -300 cmg, temperature profile- 30°C - 92°C, speed-250 rpm/min.

Evaluation: The parameters i.e., gelatinization temperature, peak viscosity, hot and cold paste viscosity, set back values and break down were evaluated
with software under window 98, 2000 and ME. The specimen curve illustrating the interpretation of the amylogram is given in the Figure 5C.2.

**Figure 5C.2: Specimen curve illustrating interpretation of amylogram**

![Specimen curve illustrating interpretation of amylogram](image)

The terms in the above Figure 5C.2 are explained as below;

**Gelatinization temperature** (°C): The temperature in ° C at which viscosity starts increasing due to gelatinization.

**Peak viscosity** (BU): The highest viscosity attained by the paste during the gelatinization process.

**Hot paste viscosity** (BU): The viscosity of the paste at 95° C.

**Cold paste viscosity** (BU): The viscosity of the cooked paste after cooling to 50° C.

**Break down** (BU): Difference between peak viscosity and hot paste viscosity.

**Set back values** (BU): The increase in viscosity on cooling from 95 to 50° C.

### 5C.3.4. Bread making characteristics

The following formulation was used for the preparation of sweet bread, wheat flour: 100 g; L-asparaginase enzyme (50, 100, 200 and 300U) separately, compressed yeast: 2 g; salt: 1.5 g; sugar: 12 g; hydrogenated fat: 1 g and water (optimum water absorption as determined with the farinograph). Control was maintained without adding enzyme. All the ingredients were mixed until fine dough was formed. Breads in quadruplicate were prepared by
mixing the ingredients in a Hobart mixer (Model N-50, Hobart, GmbH, Offenburg, Germany) with a flat blade for 4 min at 61 rpm. The dough was fermented in a chamber maintained at 30°C and 75 % relative humidity (RH) for 90 min, remixed, rounded and again fermented for 25 min, moulded, proofed for 55 min at 30°C, 85 % RH and baked for 25 min at 220°C. After baking the bread samples were removed from baking pans and placed on wire rack to cool for one hour at ambient temperature and packed.

5C.3.5. Physico-sensory and chemical characteristics of bread

5C.3.5.1. Bread weight and volume

Bread weight was recorded using instrument and bread volume was determined by the rapeseed displacement method (Chopin, S.A, France).

5C.3.5.2. Crumb firmness

Crumb firmness (method 74-09) was measured according to AACC (2000) procedure using texture analyzer (Model TA-HDi, Stable Microsystems, Surrey, UK) under the following conditions: sample thickness - 25 mm, load cell – 10 Kg, plunger diameter – 36 mm and plunger speed – 100 mm per minute. Crumb firmness, in terms of force (g) required for 25 % compression was measured.

5C.3.5.3. Sensory evaluation

Sensory evaluation of bread was carried out by 8 male and 6 female panelists ranging in age from 25-55 years. The panelists were trained in four sessions, involving 2 h of training in each session. Five samples of bread in four replicates were evaluated by each panelist for crust colour for the maximum score of 10 and shape (20); crumb colour (15), grain (20), mouthfeel (20) and taste (15) according to Indrani et al. (2003). The overall quality score (max-100) was taken as the combined score of all six quality attributes.

5C.3.5.4. Color estimation

Color estimation of untreated and L-asparaginase treated bread samples were measured as described under section (5B.4.3).
5C.3.6. Determination of Moisture

The moisture content was determined according to the American Association of Cereal Chemists (AACC) standard methods (method 44-15). Measurement was based on the principle of loss in weight after drying the sample for 1 hour at 130°C and expressed in percentage of the moisture content. 2 gm of the powdered sample was weighed in aluminium dish and placed in an hot air oven maintained at 130°C for 1 hr. Then cooled in desiccators to ambient temperature and weighed to constant weight. The percentage loss in weight is calculated using the equation 5C.2.

\[
\text{Moisture (\%)} = \frac{A-B}{A-C} \times 100 \quad \text{(Eq. 5C.2)}
\]

Where

- \(A\) = weight of flour + aluminium dish before drying
- \(B\) = weight of flour + aluminium dish after drying
- \(C\) = weight of aluminium dish.

5C.3.7. Estimation of protein

The protein content of the samples was determined by Kjeldahl method (AACC 46-12). Approximately 1 g of sample was weighed into a Kjeldahl flask. 1-2 gm of digestion mixture (5C.2.4.4), 20 ml of concentrated sulphuric acid and 2 or 3 glass beads were added to the flask and kept for digestion. The flasks were removed when the liquid became clear, cooled, diluted, transferred into a 100 ml volumetric flask and it was made up to the mark. 5 ml of clear liquid was pipetted into the distillation flask with 10 ml of 40% sodium hydroxide; steam distilled and about 25 ml of distillate containing ammonia was collected to which 5 ml of 2% boric acid containing 3 drops of mixed indicator (5C.2.4.4) was added. The ammonium metaborate was titrated with standardized N/70 hydrochloric acid. The percentage of protein was calculated using the equation 5C.3.

\[
\text{Protein (\%)} = \frac{(\text{Titer value} - \text{blank}) \times 2.7 \times 5.7}{(\text{acid value} - \text{blank})} \quad \text{(Eq. 5C.3)}
\]

In protein analysis, Kjeldahl nitrogen represents the total nitrogen content of the food also coming from non-protein components. So, a correction factor of 5.70 (for wheat samples) is used to get the % of protein. Blank determination was carried out with the sample as given above (5C.3.5.6) and the ml of N/70 hydrochloric acid required was subtracted from the sample reading.
**5C.3.8. Determination of Ash**

Total ash is the inorganic residual remaining on incineration under atmospheric pressure. Total ash was determined by the AACC standard method (method 08-01) as follows: Ten gm of sample was weighed in a pre-weighed silica dish and was then incinerated in muffle furnace maintained at 550°C-600°C until light gray ash resulted. Silica dish containing ash was cooled in the desiccator and weighed to constant weight. Amount of ash, expressed as percentage was calculated by the equation 5C.4.

\[
\text{Ash content (\%)} = \frac{(W_2 - W_1)}{(W_1 - W)} \times 100 \quad (\text{Eq. 5C.4})
\]

Where

\( W \) = weight of empty silica dish, (g)

\( W_1 \) = weight of silica dish + sample before drying, (g)

\( W_2 \) = weight of silica dish + sample after drying, (g).

**5C.3.9. Extraction and analysis of acrylamide**

AA was extracted and analysed according to the method described under (5B.4.1 and 5B.4.2.1).

**5C.3.10. HMF extraction and analysis**

HMF was extracted according to Garcia-Villanova et al. (1993) by suspending 500 mg of freeze dried bread sample in 5 ml of deionised water. The tube was shaken vigorously for 1 min and clarified with 0.25 ml each of potassium ferrocyanide (5C.2.4.1) and zinc acetate (5C.2.4.1) solutions. The resulting mixture was centrifuged at 2500 g for 10 min at 4°C. The supernatant was collected in a 10 ml volumetric flask and two further extractions were performed using 2 ml of deionised water. The extracts were filtered (0.2 µm) and analyzed by HPLC using C-18 reverse phase column, with water and acetonitrile (95:5, v/v) as mobile phase at a flow rate of 1 ml/min at 25°C under isocratic conditions. Detection was done at wavelength of 280 nm by PDA detector. HMF concentrations were calculated from the calibration curve plotted with standard solutions of HMF dissolved in Milli-Q water (0.015 to 1.5 mg/ml). All the analysis were performed in triplicate and the results are expressed as mg/kg sample.
5C.3.11. Extraction and analysis of free L-asparagine

One gram freeze dried bread sample was extracted in 25 ml of trichloroacetic acid (5C.2.4.2). After 24 h of constant shaking, the sample was filtered with Whatman no.1 filter paper, and 1 ml of the filtrate was centrifuged at 5000 rpm for 5 min at 4°C (Weining et al., 2008). Supernatant was analyzed using HPLC (Shimadzu 10AT series).

HPLC conditions were maintained according to method described by Cheng et al. (2007). L-asparagine was detected by UV detector using C-18 reverse phase column with acetonitrile: 0.03M potassium phosphate of pH 3.2 (20:80 v/v) as mobile phase at a flow rate of 0.5 ml/min. 10 µl of sample was injected and analysed at 190 nm. L-asparagine content was estimated by using external standard method within the range of 5 to 50 µg/10µl. All the analyses were performed in triplicate and the average results are expressed as mg/kg sample.

5C.3.12. Total Sugar estimation

Total sugar content of freeze dried bread samples was analyzed according to AOAC (1999). The sugar content is estimated by determining the volume of the unknown sugar solution required to completely reduce a measured volume of Fehling’s solution (5C.2.4.3). Sugar reduces the copper in Fehling’s solution to red insoluble cuprous oxide.

Standardization of Fehling’s solution was done by pouring 50 ml of standard sugar solution in burette. 5ml each of Fehling’s solution A and B was pipetted in 250ml conical flask and boiled for 2 min. At the end of 2 min, 1 ml of ethylene blue indicator solution was added without interrupting the boiling. While the contents of the flask continue to boil, standard sugar solution was added (the titration should be completed within 30 seconds, so that contents of flask continue to boil, altogether for 3 mins interruption). The titre value was noted. (i.e., the volume in ml of standard sugar solution used for the reduction of all the copper in 10 ml of Fehling’s solution). Factor to convert the values of glucose to starch was calculated by equation 5C.5.

Calculations:

\[ \text{Factor} = \frac{W}{X} \times Y \quad \text{Eq. 5C.5} \]

\[ = 0.2/100 \times \text{Total Volume.} \]
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W= weight of the sample
X=final volume of sample.
Y=volume of sugar solution require to reduce 10ml of Fehling’s solution.

5C.3.12.1. Total sugar estimation in bread samples
Twenty grams of freeze dried bread samples was taken in a 250 ml beaker and bread paste was made by adding little quantity of dist.H₂O. 5ml of 10% ammonia, 5ml of 10% acetic acid, 12.5 ml of Zinc acetate and 12.5 ml of potassium ferrocyanide were added and the mixture was left for 1 h after making volume upto 250 ml by adding dist.H₂O. The solution was filtered using Whattman no. 1 filter paper and filtrate obtained was noted. The filtrate was transferred to 250 ml volumetric flask and 1ml hydrochloric acid for every 10 ml of filtrate was added and left for inversion for 2 days. To the mixture phenolphthalein indicator was added and neutralized with sodium hydroxide pellets. If the filtrate becomes dark pink, 1:3 HCl was added to make it lighter pink. The volumetric flask was kept in cold for 15-20 mins; the mixture was again filtered using Whattman 1 filter paper and filled in burette. 25ml of dist.H₂O was taken in 250 ml conical flask and 5ml each of Fehling’s solution A and B were added, the mixture was kept on hot plate. To the boiling mixture methylene blue was added and titrated. If the titre value would be above 3, then sample was diluted dist.H₂O.

10ml of the solution $\longrightarrow$ 100ml with distilled water.
If the titre value is 6 then,
20ml of the solution $\longrightarrow$ 100ml with distilled water.
Then the burette is filled with diluted solution and titration was repeated as above and total sugars were calculated using equation 5C.6.

Calculations:
Total sugars= $\frac{250}{W} \times \frac{250}{V_1} \times \frac{100}{V_2} \times \frac{\text{Factor}}{\text{T.V}} \times 100$  \hspace{1cm} (Eq. 5C.6)

Where; W = sample weight

\hspace{1cm} V_1 = \text{volume for inversion}
\hspace{1cm} V_2 = \text{volume for dilution}
\hspace{1cm} \text{T.V} = \text{Titre value}.
The results were expressed as percentage of sugar. All samples were analyzed in triplicates and average results are expressed as percentage per gram of bread sample.

5C.3.13. Reducing sugars estimation

The content of reducing sugars in the dough samples was determined as described by Fink et al. (2006) with slight modifications as per Weining et al. (2008). 3 g of Freeze-dried and milled sample was mixed with 50 ml of distilled water and kept in water bath at 70°C for 20 mins to extract sugars into solution. The solution was cooled and centrifuged at 6000 rpm for 10 minutes. Supernatant was taken in 100ml volumetric flask and made up to 100 ml. 0.5 ml of this solution was taken and 1.5ml of DNS reagent (5C.2.4.5) was added and kept in boiling water bath for 5 mins. It was cooled, diluted by adding 20 ml of distilled water and OD was measured at 540nm. The concentration was calibrated from the glucose standard curve. All samples were analyzed in triplicate and average results are expressed as mg/g.

5C.3.14. Statistical analysis

The data pertaining to chemical characteristics were expressed as mean ± standard error. All results of bread making characteristics were analyzed using Duncan’s new multiple range test with different experiment groups appropriate to the completely randomized design with four replicates each, as described by Steel and Torrie, (1980). The significant level was established at P ≤ 0.05.

5C.4. Results and Discussion

5C.4.1. Farinograph and amylograph characteristics

The farinograph and amylograph characteristics of wheat flour as influenced by the addition of L-asparaginase (50-300 U) are illustrated in Figure 5C.3. Water absorption, which is the amount of water required for the dough to have a definite consistency, increased from 61.6 ± 0.45% in the control wheat flour to 62.0 ± 0.45 % with the addition of 50 U, 100 U (62.2 ± 0.50 %), 200U (62.4 ± 0.35 %), 300 U (62.5 ± 0.5 %) of L-asparaginase respectively. Dough stability is the time in minutes the dough remains on 500 BU consistency line. There is a marginal increase in the dough stability from
4.4 (± 0.35 min) to 5.2 (± 0.45 min) with the addition of 50-300U of L-asparaginase enzyme, however, the increase in water absorption and dough stability was not statistically significant (P ≤ 0.05).

The pasting temperature provides an indication of minimum temperature required to cook. Pasting temperature of the control wheat flour and wheat flour with added L-asparaginase (50-300 U) ranged between 65.2 and 65.4 (± 0.25°C). Peak viscosity (the highest viscosity of the paste during the heating phase) represents the ability of the starch granules to swell freely before their physical break down and also α-amylase activity. Peak viscosity of wheat flour decreased from 897-874 (±15 BU) with increase in the enzyme concentration from 0 to 300 U. These results showed that use of L-asparaginase does not have much significant (P ≤ 0.05) effect on the pasting characteristics of wheat flour. Effect of L-asparaginase on the rheological characteristics of wheat flour has not been reported yet, however Indrani et al.(2003) studied the effect of fungal alpha amylase (FA), glucose oxidase (GO), protease (PRO) and xylanase (XY) on the rheological and bread making performances of wheat flour dough. The authors opined that addition of FA or PRO decreased whereas addition of XY or GO increased the dough stability. Among different enzymes XY produced the highest improvement in volume and overall quality score. It is reported that there is no data concerning the impact of addition of bakery improvers like α-amylase, amylglucosidase and to a lesser extent protease on AA content (Claus et al., 2008).
Figure 5C.3: Influence of L-asparaginase on farinograph and amyllograph characteristics of wheat flour

<table>
<thead>
<tr>
<th>Enzyme treatment</th>
<th>Farinograph water absorption (%)</th>
<th>Pasting temperature (°C)</th>
<th>Peak viscosity (BU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat flour</td>
<td>1200</td>
<td>1000</td>
<td>800</td>
</tr>
<tr>
<td>+50 U</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>+100 U</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>+200 U</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>+300 U</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Error bars are the mean of three replicates ± standard deviation.
Black square (■), asterisk (*), number sign (#) not significant at P≤0.05.

5C.4.2. Physico-sensory characteristics of bread

The moisture content of control bread and breads with L-asparaginase ranged between 33.2 to 34.4%, ash (1.72-1.80%) and protein (9.0-9.4 %) (Table 5C.1). The data on physico-sensory characteristics of bread showed that addition of L-asparaginase (at 50-300 U levels) as a AA reducing agent did not showed any significant effect on the physico-sensory characteristics of the breads as compared to the control (Figure 5C.4). The crumb firmness, a measure of texture of control bread was 625(±15) g and it ranged between 600 to 615 (± 12) g (Figure 5C.4) for the breads treated with L-asparaginase. Bread with L-asparaginase enzyme showed darker crust colour as indicated by lower L* value than control bread without L-asparaginase enzyme (Table 5C.2). However the shape of the crust of both breads was symmetrical and convex. The brown colour formed in the crust, is a product of the Maillard Reaction between reducing sugars and proteins. The redness (a*) and yellowness (b*) of the crust was also significantly different between all samples (Table 5C.2). The crumb of the breads exhibited whitish cream colour (Figure 5C.5), fine grain, clean mouth feel and pleasant taste which are
desirable organoleptic properties of bread. The total sensory score of the control bread was 85 (± 1.5) and breads with 50-300 U L-asparaginase ranged between 82-84 (±2.0) indicating not much significant effect (P ≤ 0.05) on the bread making characteristics of wheat flour. Ciesarova et al. (2009) also observed that L-asparaginase enzyme treatment and a substitution of ammonium raising agent for sodium salt addition in ginger breads results in more than 97% reduction of AA content with no detrimental effect on sensory quality of final product.

**Table 5C.1: Physico-chemical analysis of bread**

<table>
<thead>
<tr>
<th>Sl.no</th>
<th>Enzyme treatment</th>
<th>Moisture content (%)</th>
<th>Ash content (%)</th>
<th>Protein content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>33.1</td>
<td>1.72</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>50 U</td>
<td>33.4</td>
<td>1.72</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>100 U</td>
<td>33.4</td>
<td>1.76</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>200 U</td>
<td>33.9</td>
<td>1.78</td>
<td>9.36</td>
</tr>
<tr>
<td>5</td>
<td>300 U</td>
<td>34.4</td>
<td>1.80</td>
<td>9.36</td>
</tr>
</tbody>
</table>

Data represent the mean of three replicates ± standard error.

**Figure 5C.4: Effect of L-asparaginase on volume, crumb firmness and physico-sensory characteristics of sweet bread**

*Error bars* are the mean of three replicates ± standard deviation. Black square (■), asterisk (*), number sign (#) not significant at P≤0.05.
### Table 5C.2: Bread colour analysis of the control and L-asparaginase treated breads

<table>
<thead>
<tr>
<th>Enzyme Concentration</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>61.73 ± 3.1</td>
<td>10.97 ± 0.5</td>
<td>22.84 ± 1.8</td>
</tr>
<tr>
<td>50 U</td>
<td>59.06 ± 2.6</td>
<td>10.54 ± 0.8</td>
<td>22.61 ± 1.2</td>
</tr>
<tr>
<td>100 U</td>
<td>58.53 ± 2.4</td>
<td>10.14 ± 0.4</td>
<td>22.53 ± 1.2</td>
</tr>
<tr>
<td>200 U</td>
<td>56.07 ± 3.8</td>
<td>10.08 ± 0.5</td>
<td>21.83 ± 2.1</td>
</tr>
<tr>
<td>300 U</td>
<td>55.02 ± 2.1</td>
<td>9.85 ± 1.2</td>
<td>20.23 ± 2.2</td>
</tr>
<tr>
<td>Crumb</td>
<td>77.56 ± 1.5</td>
<td>0.59 ± 0.07</td>
<td>12.96 ± 0.8</td>
</tr>
<tr>
<td>Control</td>
<td>76.23 ± 2.1</td>
<td>0.57 ± 0.05</td>
<td>12.74 ± 0.6</td>
</tr>
<tr>
<td>50 U</td>
<td>75.27 ± 1.3</td>
<td>0.55 ± 0.05</td>
<td>12.51 ± 0.8</td>
</tr>
<tr>
<td>100 U</td>
<td>74.87 ± 2.8</td>
<td>0.53 ± 0.06</td>
<td>12.49 ± 0.9</td>
</tr>
<tr>
<td>200 U</td>
<td>74.47 ± 2.1</td>
<td>0.50 ± 0.03</td>
<td>12.48 ± 0.6</td>
</tr>
</tbody>
</table>

Data represent the mean of three replicates ± standard error.

L* = lightness, higher values indicate lighter colour.

a* = redness.

b* = yellowness; higher colour intensity is indicated by higher values.

### Figure 5C.5: Photograph of bread with different levels of L-asparaginase enzyme treatment

A: Control; B: 50U; C: 100U; D: 200U; E: 300U.

### 5C.4.3. Acrylamide formation in bread

Millard reaction and caramelization are the most important chemical events occurring during the manufacture of bakery products. Among different
products produced during baking, AA and HMF have received great attention in recent years. In our experiment, L-asparaginase was mixed with the dough and allowed to convert L-asparagine present in dough so as to make it unavailable for Millard Reaction. The results of the analysis of baked bread for AA content during normal process of baking showed that, when bread was prepared with L-asparaginase treated dough AA formation was reduced. The HPLC profile of standard AA, untreated control bread and L-asparaginase treated bread are given in Figures 5C.6a, b & c. The reduction increased with increase in enzyme concentration from 50 to 300U. In untreated bread the crust portion had more AA content of 729 µg/kg and the crumb portion had 143 µg/kg which was 5 times less than the crust portion (Table 5C.3). In the dough treated with 50U of enzyme resulted in 73% reduction in AA formation. At 100 U of enzyme addition, reduction in AA formation increased to 92% in crust portion. The reduction in AA formation at higher levels was not significant i.e., only 95 and 97% reductions were observed at 200 and 300U of enzyme respectively, i.e., there was only an increase of 3 and 5 % reduction of AA (Table 5C.3) formation at these enzyme concentrations. Although the difference in reduction was low, 300 U of L-asparaginase was chosen as maximum AA reduction (97%) was obtained (Figure 5C.6c). In all the concentrations studied, the reduction was predominant in the crust region than in the crumb region. In the crumb, the AA reduction was from 52 to 71% with 50 to 300U of enzyme treatment. Claus et al. (2005) demonstrated that AA is predominantly generated in the outer crust layer where more than 99% can be found, while only trace amounts are detectable in the crumb (Sadd and Hamlet, 2005). This can be ascribed to the lower temperatures of only 100°C in the inner parts of bakery products. Therefore, Surdyk et al. (2004) assumed that AA found in the crumb originates from carry-over effects from the crust. When the enzyme preparation was added to wheat cracker production, AA levels decreased by at least 70% without any changes in the colour or flavour of the products (Vass et al., 2004). In corn-based foods, Teras et al. (2004) reported an L-asparagine reduction of up to 90%, however, details concerning the AA levels were not provided.
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Acrylamide reduction in Sweet bread

Table 5C.3: Acrylamide concentration in bread crust and crumb in L-asparaginase treated breads

<table>
<thead>
<tr>
<th>Sl.no</th>
<th>Enzyme treatment</th>
<th>Acrylamide (µg/kg) Bread Crust</th>
<th>Acrylamide (µg/kg) Bread Crumb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>729± 1.48</td>
<td>143± 1.26</td>
</tr>
<tr>
<td>2</td>
<td>50 U</td>
<td>200± 1.38</td>
<td>68± 1.18</td>
</tr>
<tr>
<td>3</td>
<td>100 U</td>
<td>60± 0.98</td>
<td>68± 1.34</td>
</tr>
<tr>
<td>4</td>
<td>200 U</td>
<td>40± 1.27</td>
<td>57± 1.08</td>
</tr>
<tr>
<td>5</td>
<td>300 U</td>
<td>24± 0.86</td>
<td>41± 0.84</td>
</tr>
</tbody>
</table>

Data represent the mean of three replicates ± standard error.

Figure 5C.6: HPLC analysis of acrylamide

a. Standard acrylamide

Standard acrylamide: retention time of 6.33 min.
b. Bread control

Bread prepared without L-asparaginase treatment.

c. L-asparaginase treated bread

Bread prepared from dough treated with 300 U of L-asparaginase.
5C.4.4. L-asparagine content in bread

In our experiments, as the enzyme concentration was increased from 50 to 300U, the free L-asparagine was reduced from 127.4 to 23.4 mg/kg. The reduction of L-asparagine content was in the range of 25 to 75% with respect to 100 to 200 U of enzyme treatment. The higher reduction of 81% of L-asparagine was observed at 300 U of enzyme treated bread (Figure 5C.7). This lower levels of L-asparagine directly corresponded to the AA reduction. The free L-asparagine content in the wheat flour was found to be 302 mg/kg. According to Fredriksson et al. (2004), L-asparagine concentrations in wholegrain wheat and rye flours were 500 and 1100 mg/kg, respectively. Hamlet et al. (2008) reported the concentration of free asparagine in wheat flour varying between 74 to 664 mg/kg, while in rye flour it ranged between 319 to 791 mg/kg (Springer et al., 2003), this shows that the L-asparagine content varies in different types of flours. So selection of flour with low L-asparagine content would be advantageous in getting bread with low AA content. The concentration of free L-asparagine in flour and dough was shown to correspond with AA formation in several bakery products according to Springer et al. (2003). The direct relationship between L-asparagine content and AA formation was similar to previous results showing Maillard reaction to be the major pathway that resulted in AA formation (Yaylayan and Stadler, 2005; Zyzak et al., 2003).
Figure 5C.7: Influence of L-Asparaginase enzyme on free L-asparagine content in bread

![Graph showing the influence of L-Asparaginase enzyme on free L-asparagine content in bread.](image)

Wheat Flour: Ingredient used in Bread making. Control: Without enzyme addition. Error bars are the mean of three replicates ± standard deviation.

5C.4.5. Sugar content in bread

Total sugar content in enzyme treated bread, crumb and crust portions are shown in the Figure 5C.8. Control bread contained 6.41 and 11.3% sugars respectively in crumb and crust. With increase in enzyme concentration there is gradual decrease in the sugar content of both crust and crumb. The content of reducing sugars in untreated bread samples was found to be 45.7 mg/g. With increase in L-asparaginase enzyme concentration there is slight decrease in the amount of reducing sugars. At 200 and 300 U level reducing sugar was found to be 34.6 mg/g and 33.4 mg/g respectively. The amounts of reducing sugars in the enzyme treated breads are shown in Table 5C.4. Although L-asparagine is the limiting factor in bakery products, sugar also plays a crucial role (Surdyk et al., 2004). It was demonstrated by Vass et al. (2004) that replacing invert sugar syrup with sucrose in wheat crackers reduced AA by 60%. Similar effects were also observed for gingerbread (Amrein et al., 2004).
Figure 5C.8: Total sugar content in crust and crumb of bread treated with L-asparaginase

Table 5C.4: Reducing sugar content of breads prepared with L-asparaginase

<table>
<thead>
<tr>
<th>Samples</th>
<th>Reducing sugar (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>45.7± 0.97</td>
</tr>
<tr>
<td>50 U</td>
<td>43.1± 0.86</td>
</tr>
<tr>
<td>100 U</td>
<td>40.6± 0.84</td>
</tr>
<tr>
<td>200 U</td>
<td>34.6± 0.92</td>
</tr>
<tr>
<td>300 U</td>
<td>33.4± 0.97</td>
</tr>
</tbody>
</table>

5C.4.6. HMF analysis

HMF is a classical chemical marker of the extent of the Maillard reaction in thermally processed foods and particularly in cereal products (Murkovic and Pichler, 2006). Figure 5C.9 showed the addition of L-asparaginase reduced the amount of HMF formation in sweet bread. HMF concentration in control bread was below the quantification limit of 0.6 mg/kg. This value is lower than HMF concentration reported (1.5 ± 0.3 mg/kg and
17.9 ± 0.7 mg/kg) in literature for bread (Ramirez-Jimenez, 1998). Edoardo et al. (2009) also reported HMF concentration in untoasted bread slices to be below the quantification limit of 0.5 mg/kg. Control bread produced lower amount of HMF, probably because of the lesser free amino acids or proteins content in wheat flour. Similar results have been obtained by Edoardo et al. (2009) who obtained low HMF in bread made out of wheat flour as compared to that with rye flour. In enzyme treated bread the HMF content decreased significantly compared with the control. At 50U of enzyme there was 74% reduction in HMF formation. The reduction increased with increase in enzyme concentration and there was 98.4% reduction of HMF formation when 300U of enzyme was used. There was great variation for HMF concentration of breads ranging from 3.4 to 68.8 mg/kg (Ruiz et al., 2004). Ramirez-Jimenez et al., (2000) have reported that conventional white breads contained 23.2 mg/kg of HMF. They have also reported HMF contents of mixed-grain breads ranging from 4.8 mg/kg for oat bread to 23.4 mg/kg for rye and wheat bran breads. HMF concentration was 46.69, 47.02, 17.25 mg/kg for rye, wheat and whole-wheat containing samples, respectively when the toasting was done at 180°C for 25 min (Edoardo et al., 2009). This variation may be attributed to the formulation change in the processing temperature and the effect of the flour type also.
Figure 5C.9: HMF formation in sweet bread treated with different levels of L-asparaginase

Control: Without enzyme addition. Error bars are the mean of three replicates ± standard deviation.
5C.5. Summary and Conclusions

- Acrylamide, a non-volatile compound, classified as “potentially carcinogenic to humans” is formed in heat-treated foods rich in carbohydrates, such as baked foods, man can be directly exposed to it by consuming these food materials.

- An application of microbial L-asparaginase in reducing acrylamide formation during the bread making process, without affecting its physico-sensory properties was attempted.

- L-asparaginase produced from *Cladosporium* sp. was treated to wheat based dough at different concentrations (50-300U).

- There was no change in the rheological properties of wheat flour and physico-sensory characteristics of bread with L-asparaginase treatment.

- Moisture, sugars, L-asparagine, acrylamide and some indicators of Millard reaction (Hydroxymethylfurfural (HMF), color, browning) were estimated.

- With increase in L-asparaginase level the acrylamide formation was reduced. At 300 U there was 97% and 73% reduction of acrylamide formation in the crust and crumb regions of bread respectively.

- HMF, a common intermediate product in the Maillard reaction and a genotoxic compound via 5-sulfoxymethylfurfural (SMF), also decreased in L-asparaginase treated bread samples.

- These results indicated the potential of L-asparaginase enzyme for industrial and domestic applications in reducing harmful Maillard reaction compounds.

- This opens avenues for the application of microbial enzymes in obtaining acrylamide free foods.

5C.6. Bibliography

Approved methods of the American Association of Cereal Chemists (AACC).


Hamlet CG, Sadd PA & Liang L. Correlations between the amounts of free asparagine and saccharides present in commercial cereal flours in the UK and the generation of acrylamide during cooking. *Journal of Agriculture and Food Chemistry.* 2008; 56(15): 6145-6153.


